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ASSESMENT OF GLUTATHIONE REDUCTASE INHIBITION

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Glutathione reductase is a crucial enzyme for maintaining of intracellular glutathione levels. This enzyme catalyzes the NADPH-dependent reduction of glutathione disulfide to the reduced form (GSH). The aim of our study was to estimate a possible inhibitory effect on glutathione reductase activity in the presence of two substances that cause glutathione depletion - ethacrynic acid and diethyl maleate. We also tested glutathione as a possible inhibitor. The experiments were performed with yeast glutathione reductase. GR activity was determined using spectrophotometric method based on measurement of absorbance decline (λ = 340 nm) due to oxidation of NADPH. We found that dose dependent inhibition of glutathione reductase occurred in the presence of ethacrynic acid; the enzyme activity was inhibited by 19 % and 29 % in the presence of 500 μM and 1000 μM ethacrynic acid, respectively. We also found that although diethyl maleate is able to induce deep glutathione depletion in the cell, it does not affect the GR activity. On the other hand, we found dose dependent inhibitory effect through reduced glutathione — the presence of 10 mM GSH caused a decrease in enzyme activity

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by 60 %. We conclude that our finding of inhibitory effect in the presence of glutathione is of great importance since the GSH levels are very high in the cells. It follows that glutathione reductase possesses a substantial reserve in enzyme activity that could be used in oxidative stress conditions.

Introduction

Glutathione (γ-L-glutamyl-L-cysteinylglycine) is a major non-protein thiol in many organisms that is involved in antioxidant defense of the cells. This tripeptide is very important in maintaining of the physiological redox status within cells. Glutathione occurs in two free forms — reduced as thiol (GSH) and oxidized as glutathione disulfide (GSSG). The ratio of these two forms is an important indicator of the oxidative stress in the body [1-3]. A crucial enzyme involved in glutathione metabolism is glutathione reductase (GR). Glutathione reductase catalyzes NADPH-dependent reduction of GSSG to GSH [4]. This reaction is very important to maintain a high ratio of GSH/GSSG occurring about 100:1 under physiological conditions, which enables glutathione to function as a reducing agent and to participate in many important functions [5,6]. Thus, the inhibition of glutathione reductase activity can lead to depletion of glutathione, rapid increase of GSSG level, and increased oxidative stress in the cell [7]. The aim of our work was to estimate a possible inhibitory effect of three compounds on activity of GR - ethacrynic acid, diethyl maleate and reduced glutathione. Ethacrynic acid (EA) is commonly used as a loop diuretic. It is a highly electrophilic compound (Fig. 1)

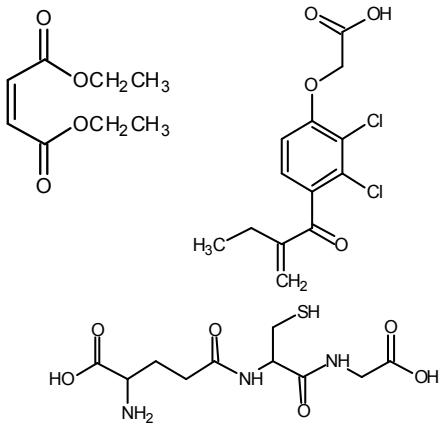

Fig. 1 Structures of ethacrynic acid, diethyl maleate and reduced glutathione

[8], which composes conjugates with glutathione either enzymatically or non-enzymatically [9,10]. Diethyl maleate (DEM) is able to induce rapid and deep glutathione depletion in the cells. It binds with GSH by a transferase reaction or

spontaneously and quickly decreases GSH levels in tissues [11-13]. However, no information about possible inhibitory effect on GR activity have been published. In addition, papers have mentioned that reduced glutathione is an inhibitor of glutathione reductase activity [5,14]. Therefore, we examined also a possible effect of GSH presence on the activity of GR.

Materials and Methods

Chemicals

Glutathione reductase (from *Saccharomyces cerevisiae*, 100 mU ml^{-1}), glutathione, glutathione disulfide, NADPH, diethyl maleate, ethacrynic acid, potassium phosphate, ethanol were purchased from Sigma-Aldrich (USA).

Determination of glutathione reductase activity

We determined the activity of glutathione reductase using spectrophotometric method based on the measurement of decline of absorbance (λ = 340 nm) due to oxidation of NADPH to $NADP^+$ (Fig. 2) ([4,15]). Briefly, we used two substrates in our assay – glutathione disulfide and NADPH. The volumes of solutions were: 50 µl GR (200 mU ml^{-1}), 25 µl GSSG (40 mM) and the assay was started by addition of 25 µl NADPH (4 mM). The decline of absorbance was monitored during 10 minutes. Results were presented as mU ml^{-1}; 1 unit (U) was defined as the amount of enzyme that catalyzes the reduction of 1 mmol GSSG per minute at pH 7.4, 25 °C. All the measurements were performed in 96-well plates using the plate reader Tecan Infinite M200.

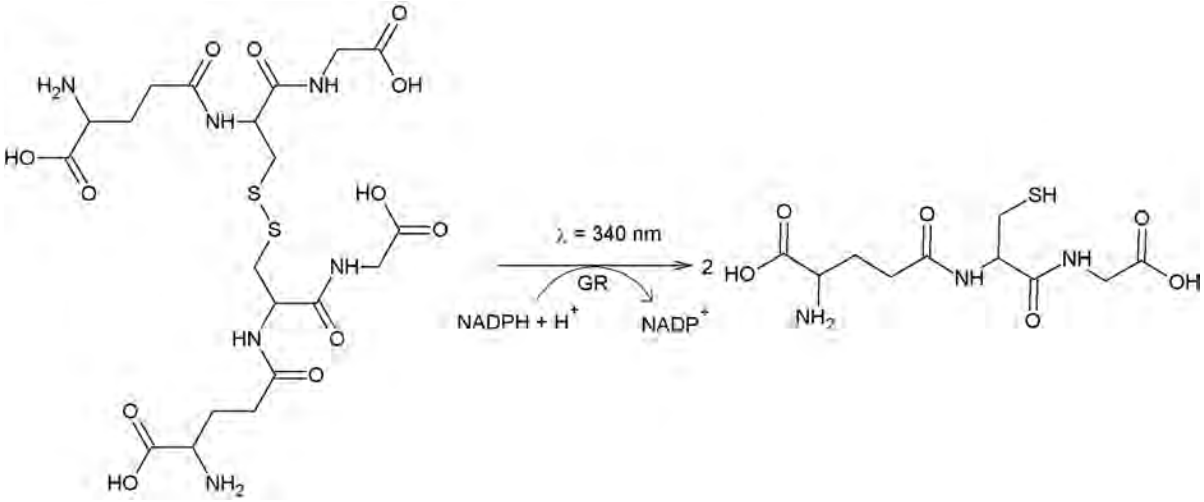

Fig. 2 Spectrophotometric assay of glutathione reductase

Estimation of GR Inhibition in the Presence of Ethacrynic Acid or Diethyl maleate

We added 10 μl of a solution (EA or DEM) to the mixture of 50 μl GR (200 mU ml^{-1}) and 25 μl of GSSG (40 mM). We prepared standard solutions of ethacrynic acid in distilled water (0-1 mM). The solution of diethyl maleate (10 mM) was prepared in ethanol. The values in the brackets mean final concentrations of compounds in wells. Control samples were prepared using the same protocol, but distilled water (10 μl) was added instead of tested solutions. The measurement was started by addition of 25 μl NADPH (4 mM) and the measurement was carried out at standard conditions noted above.

Estimation of GR Inhibition in Presence of Reduced Glutathione

We added 25 μl of a GSH solution to 50 μl of GR (200 mU ml^{-1}) and 25 μl of GSSG (40 mM) or 25 μl of distilled water in control samples. We prepared the stock solution of reduced glutathione in distilled water — tested range of GSH concentrations was 1-20 mM. The values mean the final concentrations of a compound. The measurement was started by addition of 25 μl NADPH (4 mM) and monitored spectrophotometrically again.

All the experiments were repeated twice. We calculated the enzyme activity using absorbance value. The results are expressed as the mean ± SD.

Results and Discussion

Ethacrynic acid was assessed as a possible inhibitor of glutathione reductase activity. An inhibitory effect in the presence of 63 μM, 125 μM, 250 μM, 500 μM, 1000 μM ethacrynic acid was estimated. It was found that the presence of EA caused dose dependent inhibition of glutathione reductase (Fig. 3). In the presence of 500 μM and 1000 μM ethacrynic acid, a decrease in the enzyme activity by 19 % and 29 %, respectively, was found. The data obtained are in accordance with results of other authors. The work of Hoffman *et al.* [9] stated that 500 μM EA caused the inhibition of GR activity by 93 %. The differences in the glutathione reductase inhibition level in the presence of 500 μM EA could be attributed to the use of different conditions of the assay (i.e., concentrations of substrates) and to unlike type of glutathione reductase; Hoffman *et al.* [9] used bovine glutathione reductase.

An inhibitory effect in presence of diethyl maleate was also estimated. Diethyl maleate is able to induce deep glutathione depletion in the cells [12,16,17] so that a provement of coincident inhibitory effect on glutathione reductase activity would be crucial for understanding of its toxic effect. Only one concentration of

diethyl maleate (10 mM), that is sufficient to induce a cell death [11,13,18], was tested. At this concentration, however, no inhibition of GR activity occurred; the activity of glutathione reductase was 84.0 ± 1.1 mU ml^{-1} (control activity was 83.3 ± 0.2 mU ml^{-1}).

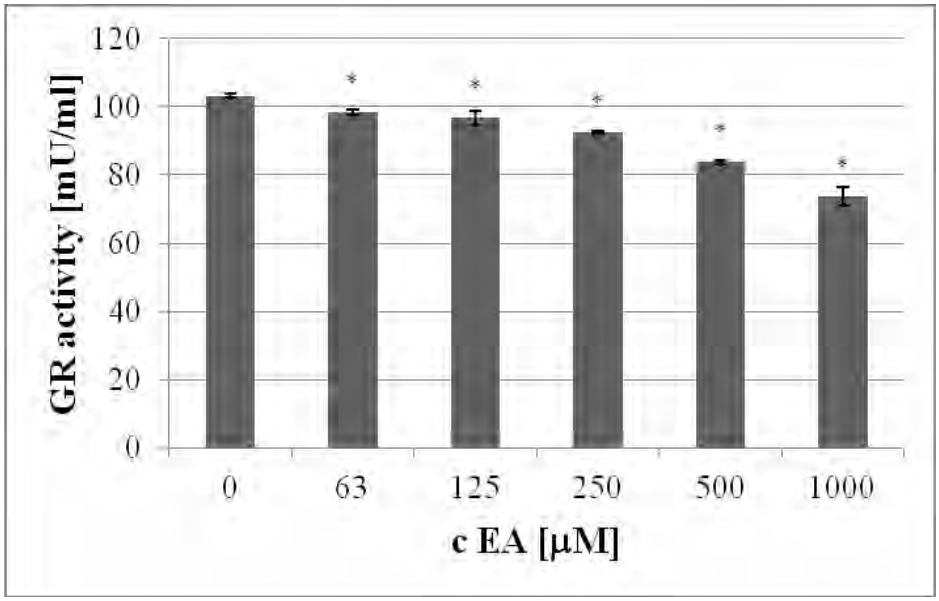

Fig. 3 Inhibitory effect of ethacrynic acid. Samples were tested in the presence of various concentrations of ethacrynic acid (63, 125, 250, 500 and 1000 μM). Conditions of assay: 9.1 mM GSSG, 0.9 mM NADPH, 100 mU ml^{-1} GR, 21 °C. The values mean the final concentrations of a compound. Decrease in absorbance (λ = 340 nm) was measured after addition of NADPH for 10 minutes. Results are expressed as mean ± SD (n = 2; *, $p < 0.05$, compared to control)

The reduced glutathione was tested to induce enzyme inhibition too. Some scientific publications showed that GR activity was significantly inhibited at physiological concentrations of reduced glutathione [5,14]. Since this finding could be of great importance, we focused on detailed description of possible inhibition of glutathione reductase in the presence of GSH (1-20 mM). It wasfound that GR activity was decreasing in relation to increasing concentrations of GSH (Fig. 4). The eight milimolar GSSG in the enzymatic assay was used. It was found that 10 mM GSH caused 60 % of inhibition in the presence of GSH/GSSG ratio nearly 1:1. Since the ratio of GSH/GSSG in the cell is 100:1 under physiological conditions, the glutathione reductase activity should be strongly inhibited at these conditions.

Conclusion

It was found that the activity of glutathione reductase was changed only in the presence of reduced glutathione and ethacrynic acid. Diethyl maleate, though a potent intracellular glutathione depletor, has no effect on the GR activity. In

500 μM ethacrynic acid, almost 20 % inhibition of the activity was found. Our main outcome is that 10 mM GSH, i.e. concentration usually occuring in the cells, is able to decrease the GR activity significantly. This phenomenon might be of great importance especially in oxidative stress conditions when the ratio of GSH to GSSG decreases and the reserve of GR activity is used.

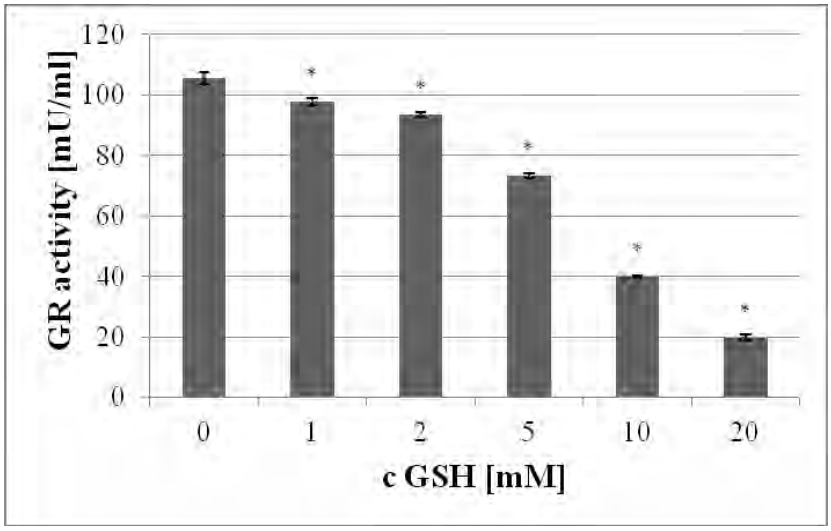

Fig. 4 Inhibitory effect of reduced glutathione. Samples were tested in the presence of different concentrations of GSH concentrations (1, 2, 5, 10 and 20 mM). Conditions of assay: 8 mM GSSG, 0.8 mM NADPH, 100 mU ml^{-1} GR, 21 °C. The values mean the final concentrations of a compound. Decrease in absorbance (λ = 340 nm) was measured after addition of NADPH for 10 minutes. Results are expressed as mean ± SD (n = 3; *, $p < 0.05$, compared to control)

Abbreviations

DEM, diethyl maleate; EA, ethacrynic acid; GR, glutathione reductase; GSH, glutathione (reduced form); GSSG, glutathione disulfide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

Acknowledgements

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